Association of *EPAS1* Gene rs4953354 Polymorphism with Susceptibility to Lung Adenocarcinoma in Female Japanese Non-Smokers

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Introduction: Hypoxia-inducible factor-2α (also called endothelial periodic acid–Schiff domain protein 1 [EPAS1]) seems to play an important role in some carcinogenesis, though there is no information on the relationship between single nucleotide polymorphism of *EPAS1* and lung cancer development. The aim of this study was to explore a possible association of the *EPAS1* gene rs4953354 polymorphism with susceptibility to lung cancer.

Methods: A case–control study of 346 patients with non–small-cell lung carcinoma (adenocarcinoma = 249, squamous cell carcinoma = 97) and 247 healthy control subjects was carried out. *A*/*G* polymorphism within an intron 2 of the *EPAS1* (rs4953354) was determined by direct sequencing.

Results: A frequency of lung adenocarcinoma patients with a minor allele *G* (*A*/*G* or *G*/*G* genotype) at the rs4953354 was much higher than that of controls (odds ratio, 1.800; 95% confidence interval, 1.161–2.791; $p = 0.008$). This association was more evident when analyzed using female never-smokers (odds ratio, 3.31; 95% confidence interval, 1.21–9.01; $p = 0.017$). Mutations in epidermal growth factor receptor tended to be frequent in patients with *G* allele at the rs4953354, compared with those with other genotypes.

Conclusion: The *EPAS1* rs4953354 may be a potentially susceptible marker for development of lung adenocarcinoma, especially in female never-smokers.

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Evaluation of the genetic factors underlying the development of lung cancer can elucidate the etiology of the disease and identify high-risk individuals for targeted screening and/or prevention.

The transcription factor hypoxia-inducible factor-α (HIF-α) plays an important role in the regulation of hypoxic signaling. It has also been reported to be involved in the progression of solid tumors and associated with various aspects of carcinogenesis, including tumor metabolism, angiogenesis, and metastasis.[1] Although HIF-1α and HIF-2α (also called endothelial periodic acid–Schiff domain protein 1 [EPAS1]) have similar functions in vitro, several recent in vivo studies on the pathophysiological roles of HIF-2α have described its role in angiogenesis during human fetal lung development, especially in the last phases of pregnancy, preparing the fetus for extrauterine life.[2,3] In addition, high expression of HIF-2α is reported to be associated with poor outcome in various cancers, including lung cancer.[4] Several groups have recently reported associations of polymorphisms (SNPs) of HIF-2α gene (*EPAS1*) with the development of renal cell carcinoma[5] and prostate cancer.[6] However in SNPs, whether HIF-2α is involved in the lung carcinogenesis remains elusive at present.

In the present study, we focused on the *EPAS1* (HIF-2α) gene rs4953354 polymorphism that has been reported to contribute to adaptation to high-altitude hypoxia in Sherpas.[7] We conducted a case–control study to explore a possible association of the rs4953354 polymorphism with susceptibility to non–small-cell lung cancer in Japanese population, with a special emphasis on adenocarcinoma developed among female never-smokers.

PATIENTS AND METHODS

Study Subjects

The study subjects were 593 individuals, including 346 patients with lung cancer and 247 healthy control subjects. The patient group included 249 patients with lung adenocarcinoma and 97 with squamous cell carcinoma, diagnosed at Shimane

University Hospital, Higashihiroshima Medical Center, and Hiroshima University Hospital, between 2009 and 2012. The healthy control subjects were randomly selected from subjects who received annual health screening at the Shimane Institute of Health Science and Hiroshima University Faculty of Medicine between 2009 and 2012. The smoking status was obtained from a self-administered questionnaire that focused on cigarette smoking history, and the subjects were divided into never-smokers, ever-smokers, including present- and ex-smokers, and those having undetermined smoking history. Table 1 lists the clinicopathological features of the patient group and anthropometric features of the control group. Each participant provided written informed consent for the collection of blood and/or tissue samples and subsequent analysis. Ethical approval was obtained from the Institutional Review Board of Shimane University Faculty of Medicine, Higashihiroshima Medical Center, and Hiroshima University Faculty of Medicine.

DNA Extraction, Genotyping, and Mutation Analysis

Genomic DNA was isolated from peripheral total blood cells or surgically resected normal tissues adjacent to cancers using NucleoSpin Blood or Tissue (MACHEREY-NAGEL, Düren, Germany) according to the instructions provided by the manufacturer. Polymerase chain reaction was performed to amplify the human *EPAS1* gene using specific primer set and conditions. The following primer set was used to amplify a 281-base pair fragment of the *EPAS1*, which contains polymorphic allele *A*/*G* (rs4953354): forward 5′-CTG GGA AAG AGG GAA TCC AGT GTG3′ and reverse 5′-CTC AGC CCA CTG TTC TCT CTT TGC-3′. The polymerase chain reaction conditions were 40 cycles of denaturation at 95°C for 30 seconds, annealing at 63°C for 30 seconds and extension at 68°C for 30 seconds. Direct sequence analysis was carried out using these primers with Big Dye Terminator Cycle Sequencing Kit and ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

TABLE 1. Clinical Characteristics of Patients and Control Subjects

	Control	Case	*p* Value
Age (yr), mean ± SD	64.0 ± 13.0	67.3 ± 10.1	0.0009[a]
Gender			
Males (*n*)	195	263	0.078[b]
Females (*n*)	52	83	
Smoking status			
Never-smoker (*n*)	71	62	0.57[b]
Ever-smoker (*n*)	168	212	
No information (*n*)	8	72	
Histopathology			
Adenocarcinoma (*n*)		249	
Squamous cell carcinoma (*n*)		97	
EGFR mutation status			
Wild (*n*)		210	
Mutated (*n*)		54	
No data available (*n*)		82	

[a]Welch's *t* test.
[b]Pearson's χ^2 test.

Statistical Analysis

All the statistical analyses were conducted, unless otherwise specified, using JMP 9.0.3 (SAS Institute, Cary, NC). We used the statistical language and environment R for calculation of extended Fisher's exact test for 2×3 tables.[8] *p* values less than 0.05 were considered statistically significant.

RESULTS

Association of *EPAS1* rs4953354 Polymorphism with Lung Cancer

The control subjects were slightly younger than the patients, while no difference was observed for gender distribution and smoking status (Table 1). The distribution of *A*/*G* alleles at rs4953354 among the control subjects fulfilled the Hardy–Weinberg equilibrium (Table 2).

We then stratified the patient group by histopathology and examined an association between the polymorphism and each lung cancer type. We found a statistically significant association between the rs4953354 and susceptibility to lung adenocarcinoma: The proportion of adenocarcinoma patients with at least one *G* allele was much higher than that of the control subjects (odds ratio [OR], 1.607; 95% confidence interval [95% CI], 1.039–2.484; $p = 0.032$). In addition, the frequency of *G* allele was much higher in adenocarcinoma patients than the control subjects (OR, 1.632; 95% CI, 1.097–2.428; $p = 0.015$). However, no such association was found for squamous cell carcinoma (Table 2).

Association of Adenocarcinoma with the SNP (rs4953354) According to Smoking Status

As genesis of lung adenocarcinoma differed between smokers and nonsmokers, we analyzed an association of the rs4953354 SNP and lung adenocarcinoma development by smoking status. The proportion of patients who were never-smokers and had at least one *G* allele was significantly higher than never-smoker control subjects (OR, 2.40; 95% CI, 1.08–5.36; $p = 0.030$). In addition, the frequency of the *G* allele among never-smoker patients was much higher than never-smoker control subjects (OR, 2.29; 95% CI, 1.12–4.65; $p = 0.020$).

Association of Adenocarcinoma with the SNP in Female Never-Smokers

Recently, lung adenocarcinoma developed in female non-smokers of eastern Asian countries attracted attention. We analyzed the association of the SNP and the adenocarcinoma susceptibility by gender. A statistically significant association was found in women (OR, 2.31; 95% CI, 1.00–5.33; $P = 0.046$, Table 3), but not in men. The same association was confirmed even after adjustment for smoking status and age: Genotype with at least one *G* allele showed a statistically significant association (adjusted OR, 3.868; 95% CI, 1.381–12.078; $p = 0.009$; Table 3).

We next analyzed the association only in female never-smokers and also found a statistically significant association

TABLE 2. Distribution of *EPAS1* rs4953354 Polymorphism in Patients and Control Subjects

	rs4953354	Control	Case	Odds Ratio (95% CI)	*p* Value
All lung cancer	*A*/*A* (*n*)	204	267		0.174[a]
	A/*G* (*n*)	41	71		0.068[b]
	G/*G* (*n*)	2	8		
	A/*A* (*n*)	204	267		0.107[c]
	A/*G* or *G*/*G* (*n*)	43	79		
	A allele (*n*)	449	605		0.062[c]
	G allele (*n*)	45	87		
Adenocarcinoma	*A*/*A* (*n*)	204	186		0.054[a]
	A/*G* (*n*)	41	56		0.017[b]
	G/*G* (*n*)	2	7		
	A/*A* (*n*)	204	166	1.800 (1.161–2.791)	0.008[c]
	A/*G* or *G*/*G* (*n*)	43	63		
	A allele (*n*)	449	428	1.632 (1.097–2.428)	0.015[c]
	G allele (*n*)	45	70		
Squamous cell carcinoma	*A*/*A* (*n*)	204	81		0.944[a]
	A/*G* (*n*)	41	15		0.887[b]
	G/*G* (*n*)	2	1		
	A/*A* (*n*)	204	81	0.937 (0.500–1.758)	0.840[c]
	A/*G* or *G*/*G* (*n*)	43	16		
	A allele (*n*)	449	177	0.958 (0.534–1.719)	0.887[c]
	G allele (*n*)	45	17		

[a]Extended Fisher's exact test.
[b]Cochran–Armitage test for trend.
[c]Pearson's χ^2 test.
CI, confidence interval.

TABLE 3. Relation between Adenocarcinoma and *EPAS1* rs4953354 Polymorphism by Gender

Sex	rs4953354	Control	Case	OR	95% CI	*p* Value	Adjusted OR (95% CI)[e]	*p* Value
Males	*A*/*A* (*n*)	162	137			0.186[a]		
	A/*G* (*n*)	32	31			0.174[b]		
	G/*G* (*n*)	1	5					
	A/*A* (*n*)	162	137	1.29	(0.76–2.18)	0.340[c]	1.255 (0.721–2.180)	0.421[d]
	A/*G* or *G*/*G* (*n*)	33	36					
	A allele (*n*)	356	305	1.41	(0.87–2.27)	0.161[c]		
	G allele (*n*)	34	41					
Females	*A*/*A* (*n*)	42	49			0.123[a]		
	A/*G* (*n*)	9	25			0.065[b]		
	G/*G* (*n*)	1	2					
	A/*A* (*n*)	42	49	2.31	(1.00–5.33)	0.046[c]	3.868 (1.381–12.078)	0.0094[d]
	A/*G* or *G*/*G* (*n*)	10	27					
	A allele (*n*)	93	123	1.99	(0.95–4.20)	0.066[c]		
	G allele (n)	11	29					

[a]Extended Fisher's exact test.
[b]Cochran–Armitage test for trend.
[c]Pearson's χ^2 test.
[d]Logistic regression analysis.
[e]Adjusted for age and smoking status.
CI, confidence interval; OR, odds ratio.

(OR, 3.31; 95% CI, 1.21–9.01; $p = 0.017$); the frequency of *G* allele was significantly higher in the patient group than the control group (OR, 2.71; 95% CI, 1.12–6.53; $p = 0.023$, Table 4). However, no such association was observed in men (Table 3).

Association of the SNP with *EGFR* Somatic Mutation in Adenocarcinoma

EGFR mutation is often found in lung adenocarcinoma in female non-smokers. We then investigated the association of the SNP with *EGFR* mutation status in 218 lung adenocarcinoma patients. The proportion of patients with at least one *G* allele tended to be higher in those with *EGFR* mutations than those with *A*/*A* homozygote (OR, 1.89; 95% CI, 0.96–3.68; $p = 0.061$, Table 5).

DISCUSSION

To the best of our knowledge, this is the first study that identified a potential genetic susceptible to lung adenocarcinoma in female never-smokers. We found that the *G* allele at the rs4953354 locus of *EPAS1* polymorphism was associated with an elevated risk of lung adenocarcinoma in female never-smokers. Lung cancer developed among never-smokers represents a unique subset of the cancer. Approximately 10% to 15% of lung cancers are diagnosed in never-smokers.[9] Accumulating evidences suggest that lung cancer developed from never-smokers is a distinct disease, with a different etiology and natural history from the one developed from smokers.[10] Although the mechanisms underlying the high risk was not determined, EPAS1 (HIF-2α) may contribute to lung carcinogenesis through the HIF signaling pathway, such as antiapoptosis, tumor angiogenesis, modified energy metabolism, and genomic instability.[1] Interestingly, our results showed that the frequency of adenocarcinoma patients with at least one *G* allele tended to be higher in those with *EGFR* mutations. Although there is no report examining the relationship between *EGFR* mutations and HIF-2α, a positive regulatory loop between EGFR and HIF signals may exist.[11] Putra et al[12] suggested that variant alleles of *HIF1A* enhanced HIF-1α functions, including its inhibitory effect on the DNA repair system, resulting in genomic instability and increased mutation of tumor suppressor genes. Database analysis showed that the rs4953354 is located close to the binding sites of several transcription factors, as identified by the chromatin immunoprecipitation sequence, suggesting that the SNP might affect *EPAS1* expression. Altered *EPAS1* activity by the *G* allele at the rs4953354 might be involved in not only activated HIF signals but also induced *EGFR* mutations, which could increase the susceptibility to lung adenocarcinoma. Thus, our study identified a potentially useful biomarker for high-risk individuals, i.e., female never-smokers, for lung adenocarcinoma. The results also enhance our understanding of the molecular mechanisms of lung carcinogenesis in such patients.

Our study has some limitations. Because the majority of the control subjects were recruited from annual health examinations, the mean age of control subjects was slightly younger than that of the patients and a detailed smoking history of control subjects such as Brinkman index was not available. Furthermore, the number of female never-smokers with lung adenocarcinoma was relatively small. To verify the relationship between the SNP and *EGFR* mutation, recruitment of a larger number of lung adenocarcinoma patients in female never-smokers will absolutely be needed.

TABLE 4. Relation between Adenocarcinoma and *EPAS1* rs4953354 Polymorphism among Female Never-Smokers

rs4953354	Control	Case	Odds Ratio (95% CI)	*p* Value
A/*A* (*n*)	36	28		0.035[a]
A/*G* (*n*)	6	16		0.028[b]
G/*G* (*n*)	1	2		
A/*A* (*n*)	36	28	3.31 (1.21–9.01)	0.017[c]
A/*G* or *G*/*G* (*n*)	7	18		
A allele (*n*)	78	72	2.71 (1.12–6.53)	0.023[c]
G allele (*n*)	8	20		

[a]Extended Fisher's exact test.
[b]Cochran–Armitage test for trend.
[c]Pearson's χ^2 test.
CI, confidence interval.

TABLE 5. Association of *EGFR* Status and *EPAS1* rs4953354 Polymorphism

	EGFR Status					
rs4953354	Wild	Mutation	OR (95% CI)	*p* Value	Adjusted OR (95% CI)[e]	*p* Value
A/*A* (*n*)	127	32		0.081[a]		
A/*G* (*n*)	34	18		0.164[b]		
G/*G* (*n*)	6	1				
A/*A* (*n*)	127	32	1.89 (0.96–3.68)	0.061[c]	2.092 (0.936–4.640)	0.072[d]
A/*G* or *G*/*G* (*n*)	40	19				
A allele (*n*)	288	82	1.53 (0.86–2.73)	0.15		
G allele (*n*)	46	20				

[a]Extended Fisher's exact test.
[b]Cochran–Armitage test for trend.
[c]Pearson's χ^2 test.
[d]Logistic regression analysis.
[e]Adjusted for age and smoking status.
CI, confidence interval; OR, odds ratio.

CONCLUSION

In conclusion, the present study identified a potential risk factor for lung adenocarcinoma in female never-smokers. A future study with a large scale is required to confirm the observation.

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